

**REMARKS**

This response is in reply to the final Office Action dated October, 2007. In the instant amendment, Claims 2-9 and 19-23 have been canceled without prejudice. Claims 1, 10, 11, 16 and 18 have been amended. Upon entry of the instant amendment, Claim 1, 10-11, 13-18 24-25 and 27-29 will be pending and under consideration.

**I. AMENDMENTS TO THE SPECIFICATION**

The specification has been amended at paragraphs [0244]-[0245] at page 70 to correct typographic error as to the figure number. The specification has been further amended at paragraph [0247] at page 70 to insert a sequence identification number.

Applicant submits that these amendments do not introduce any new matter and are fully supported by the specification as originally filed. Entry and consideration of these amendments are therefore respectfully requested

**II. AMENDMENTS TO THE CLAIMS**

Claims 2-9, 12, 19-23 and 26 have been canceled without prejudice to Applicants' right to pursue the canceled subject matter in one or more related patent applications.

Claims 1, 10, 11, 16 and 18 have been amended. Support for the amendments to Claim 1 can be found, for example, in the specification, at paragraph [0092] at page 22, paragraph [0096] at pages 23-24. Support for the amendments to Claim 18 can be found, for example, in the specification, at paragraph [0104] at page 26, and paragraph [0107] at pages 26-27.

Applicant submits that these amendments do not introduce any new matter and present the rejected claims in a better form for consideration on appeal pursuant to 37 C.F.R. § 1.116(b)(2). Accordingly, entry and consideration of the amendments are respectfully requested.

**III. CLAIM REJECTION UNDER 35 U.S.C. § 112, FIRST PARAGRAPH**

Claims 1-11, 13-25 and 27-29 stand rejected under 35 U.S.C. § 112, first paragraph, allegedly as failing to comply with the enablement requirement. Claims 2-9, 12, 19-23 and 26 have been canceled and the rejection is moot in view of their cancellation.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art

without undue experimentation. *U.S. v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). The Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. Manual of Patent Examining Procedure (hereafter “MPEP”) § 2164.04, (citing *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993)). Furthermore, “[a] specification disclosure...must be taken as being in compliance with the enablement requirement ...unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.” *Id.* (emphasis added).

The Patent Office alleges that the specification is not enabling because it fails to describe an inhibitor that reduces the interaction between a G protein-coupled receptor associated sorting protein 1 (“GASP1”) polypeptide and a G protein-coupled receptor (“GPCR”). See the Office Action, pages 2-3. Although Applicants do not agree with the Patent Office’s allegation, to advance the prosecution of the instant application, Applicants have amended Claims 1, 10, 11, 16 and 18. Claim 1 has been amended to recite a method of inhibiting agonist-induced down-regulation of a GPCR comprising contacting cells comprising the GPCR with a GASP1 polypeptide comprising the amino acid sequence of SEQ ID NO: 8 in an amount sufficient to reduce agonist-induced down-regulation of the GPCR in the cell. Claims 10-11 and 13-17 depend from Claim 1. Claim 18 has been amended to recite a method of enhancing agonist-induced down-regulation of a GPCR comprising contacting cells comprising the GPCR with a GASP1 polypeptide comprising the amino acid sequence of SEQ ID NO:2 in an amount sufficient to increase agonist-induced down-regulation of the GPCR. Claims 24-25 and 27-29 depend from Claim 18. These claims are examined to the extent that they are for *in vivo* use according to the Restriction Requirement dated June 19, 2007.

Applicants respectfully submit that Claims 11, 10-11, 13-18, 24-25 and 27-29 as amended are enabled because no undue experimentation is required for those of skill in the art to make or use the claimed invention based on the disclosure in the present application, coupled with information known in the art. The specification provides sufficient guidance for how to make and use the claimed invention. For example, the specification provides the sequence of GASP1 polypeptides. See the specification, page 28, paragraph [0113]. The specification further teaches how to make and produce the GASP1 polypeptides by synthetic or recombinant techniques. See the specification, pages 34-35, paragraphs [0122]-[0129]. The specification teaches that GASP1 polypeptides can bind the cytoplasmic tail of delta

opioid receptor (“DOR”) *in vivo*. See the specification, pages 69-71, paragraphs [0244]-[0246]; Figures 2-3. The specification further provides data to show the function of GASP1 polypeptides in mediating sorting of DOR to lysosomes. See the specification, pages 71-73, paragraphs [0248]-[0250]. As measured by radioligand binding, agonist-induced down regulation of DOR was significantly enhanced both in rate and in extent in cells overexpressing GASP 1 polypeptides comprising the sequence of SEQ ID NO:2. See the specification, pages 72-73, paragraph [0250], Figure 4(F). Agonist-induced down regulation of DOR was significantly inhibited in cells overexpressing GASP 1 polypeptides comprising the sequence of SEQ ID NO:8. See the specification, pages 71-72, paragraphs [0249]-[0250], Figure 4(E). The specification then confirms that this GASP sorting function is general to a larger class of GPCRs by showing that GASP 1 polypeptides can bind the cytoplasmic tail of numerous GPCRs such as dopamine D4 receptor, B2AR *etc.* See the specification, pages 73-74, paragraphs [0251]-[0252], Figure 5. Therefore, based on the teachings in the specification, it is clear that a sufficient guidance is provided in the specification so as to allow those of ordinary skill in the art to make and use the claimed invention. Accordingly, Applicants respectfully submit that no undue experimentation is required for make or use Claims 1, 10-11, 13-18, 24-25 and 27-29, and that these claims are fully enabled by the specification

The Patent Office alleges that the claimed invention is not enabled because the application does not provide a single *in vivo* working example of the claimed methods. Applicants respectfully disagree.

Applicants respectfully remind the Patent Office that a rigorous or an invariable exact correlation between *in vitro* or *in vivo* animal model assays and a claimed method of use is not required. See *Cross v. Iizuka*, 224, U.S.P.Q. 739, 747 (Fed. Cir. 1985). The test is whether those of skill in the art would accept the test as reasonably correlating to the claimed method. See *In re Brana* 34 U.S.P.Q.2d 1436, 1441 (Fed. Cir. 1995). In addition, since the initial burden is on the Patent Office to give reasons for the lack of enablement, the Patent Office must give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. See *Cross v. Iizuka*, 224, U.S.P.Q. 739, 747 (Fed. Cir. 1985).

The specification discloses data to show that GASP 1 polypeptides can modulate agonist-induced down regulation of PCR by regulating endocytosis of GPCRs in cell lines overexpressing GASP 1 polypeptides. See the specification, Example 1. It was known in the art that peptides that are capable of regulating endocytosis of GPCRs, such as enkephalin, can

also regulate endocytosis of GPCRs *in vivo*. See He *et al.*, 2002, *Cell* 108(2):271-282. Therefore, those of skill in the art would accept *in vitro* assays showing the ability of GASP 1 polypeptides to modulate agonist-induced down regulation of GPCRs as reasonable correlating with *in vivo* use of GASP 1 polypeptides as recited by Claims 1-11, 13-25 and 27-29.

Accordingly, it is respectfully requested that the rejection Claims 1-11, 13-25 and 27-29 under 35 U.S.C. § 112, first paragraph, be withdrawn.

**CONCLUSIONS**

In light of the above amendments and remarks, the Applicants respectfully request that the Patent Office reconsider this application with a view towards allowance.

No fees, other than that for the Petition of Extension of Time, are believed to be due with this paper. However, the Commissioner is hereby authorized to charge any required fee to Jones Day Deposit Account No. 50-3013 (order no. 405435-999011).

Respectfully submitted,

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**Exhibit A**

# Regulation of Opioid Receptor Trafficking and Morphine Tolerance by Receptor Oligomerization

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## Summary

The utility of morphine for the treatment of chronic pain is hindered by the development of tolerance to the analgesic effects of the drug. Morphine is unique among opiates in its ability to activate the mu opioid receptor (MOR) without promoting its desensitization and endocytosis. Here we demonstrate that [D-Ala<sup>2</sup>-MePhe<sup>4</sup>-Gly<sup>5</sup>-ol] enkephalin (DAMGO) can facilitate the ability of morphine to stimulate MOR endocytosis. As a consequence, rats treated chronically with both drugs show reduced analgesic tolerance compared to rats treated with morphine alone. These results demonstrate that endocytosis of the MOR can reduce the development of tolerance, and hence suggest an approach for the development of opiate analogs with enhanced efficacy for the treatment of chronic pain.

## Introduction

Opioid receptors belong to the large superfamily of G protein-coupled receptors (GPCRs). As a class, GPCRs are of fundamental physiological importance because they mediate the physiological actions of the majority of known neurotransmitters and hormones. Opioid receptors are particularly intriguing members of this receptor family because they are activated both by endogenously produced opioid peptides and by exogenously administered opiate drugs (Hughes and Kosterlitz, 1983), which are the most effective analgesics known, as well as highly addictive drugs of abuse. While opiates such as morphine remain the analgesic of choice in many cases, a major limitation to their long-term use is the development of tolerance, a profound decrease in analgesic effect observed in most patients during prolonged administration of opiate drug. In addition, long-term use of opiates causes physical dependence in some patients, a requirement for continued administration of increasing doses of drug to prevent the development of symptoms of opiate withdrawal. Despite considerable progress, the molecular and cellular mechanisms mediating the development of tolerance and dependence to morphine remain controversial (Nestler, 1996, 2001; Williams et al., 2001).

Studies using knockout mice confirm that opiate anal-

gesia and dependence are mediated by mu opioid receptors (MORs) (Matthes et al., 1996). Following activation by either alkaloid or peptide agonist, opioid receptors are regulated by multiple mechanisms, including a well-characterized and highly conserved process involving receptor phosphorylation by G protein coupled receptor kinase (GRK) and subsequent arrestin recruitment (reviewed in Ferguson, 2001). These processes can contribute directly to GPCR desensitization by facilitating the uncoupling of receptor from G protein. Following this desensitization, receptors are often endocytosed into an intracellular compartment, from which they can be recycled to the membrane, leading to receptor resensitization, or targeted for degradation, leading to receptor downregulation (Lefkowitz et al., 1998). Hence, these processes can contribute directly to tolerance by decreasing the number of functional cell surface receptors. Consequently, the prevailing view is that opioid receptor desensitization and endocytosis contribute directly to physiological tolerance by reducing the number of functional receptors present. However, morphine-activated MORs elude GRK phosphorylation and subsequent arrestin binding and desensitization (Blake et al., 1997; Whistler and von Zastrow, 1998; Zhang et al., 1998). Additionally, morphine fails to promote endocytosis of the wild-type MOR in cultured cells (Arden et al., 1995; Keith et al., 1996) and native neurons (Keith et al., 1998; Sternini et al., 1996), whereas endogenous peptide ligands, such as endorphins, and several opiate drugs, such as methadone, readily drive receptor endocytosis (Trapaidze et al., 2000). Furthermore, numerous studies have demonstrated no substantial downregulation in the number of MORs, even in profoundly tolerant animals (for example, De Vries et al., 1993; Simantov et al., 1984; reviewed in Williams et al., 2001). Hence, it is unlikely that tolerance to morphine is mediated solely by desensitization and downregulation of the receptor.

We propose a different hypothesis for the cell biological mechanisms underlying the development of tolerance to opiate drugs that is fully consistent with the available data and suggests an approach to the pharmacotherapy of chronic pain. We propose that the regulation of opioid receptors by endocytosis serves a protective role in reducing the development of tolerance to opiate drugs specifically because this mechanism regulates signaling in a rapid and reversible way. The ability of an agonist to promote endocytosis of the MOR is not linearly related with agonist activity (Whistler et al., 1999), indicating that MOR endocytosis is an independent functional property. Since agonist activity and receptor endocytosis have opposing effects on receptor-mediated signaling, the net amount of signal transmitted to the cell is a function of both processes, a relationship we term RAVE for relative activity versus endocytosis (Whistler et al., 1999). Thus, morphine would have a particularly high RAVE value as a consequence of its inability to promote receptor desensitization and endocytosis. Endorphins and opiate drugs that acutely signal with similar efficacy, yet induce receptor desensitization

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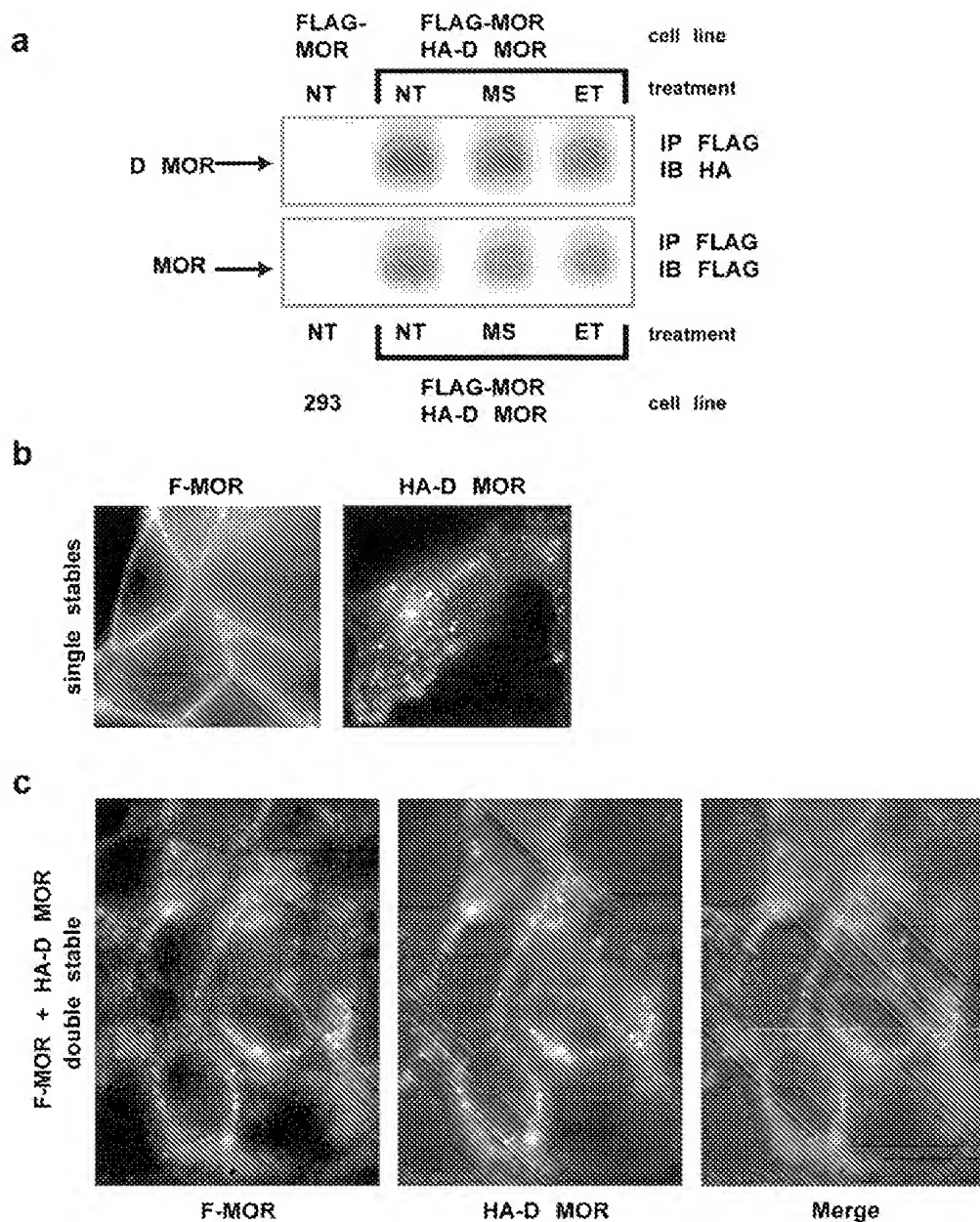


Figure 1. MORs Oligomerize in HEK 293 Cells, and this Affects Receptor Trafficking

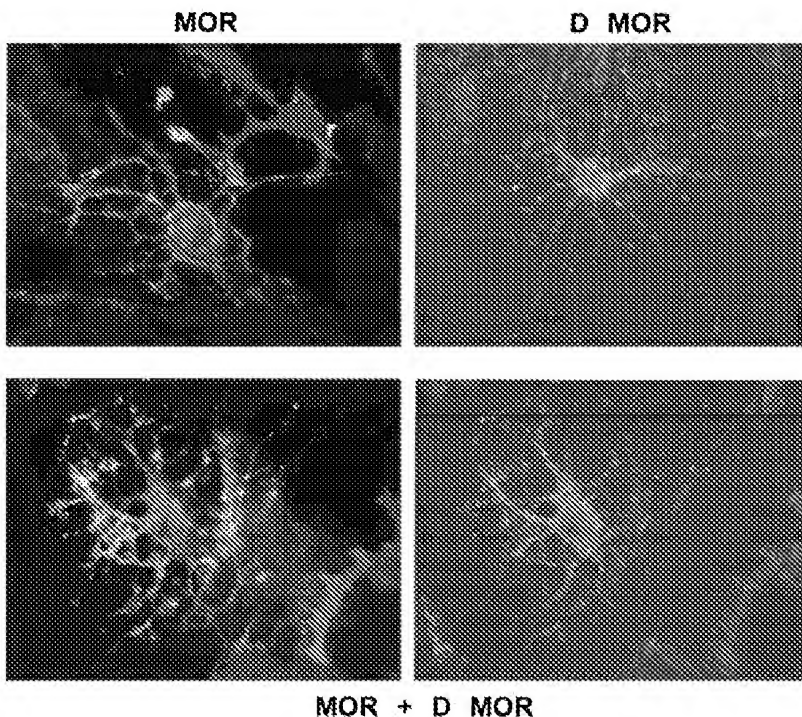
(a) Cells stably expressing both FLAG-MOR and a chimeric HA-D MOR were treated with morphine (MS), etorphine (ET), or left untreated (NT). Cells were permeabilized and receptors were immunoprecipitated with anti-FLAG antibodies, resolved by SDS-PAGE, and transferred. Oligomers were detected by immunoblotting with antibodies directed against the HA tag of the D MOR receptor (upper panel). As controls, an aliquot of the immunoprecipitate was also immunoblotted with anti-FLAG antibodies (lower panel). Cells expressing only FLAG-MOR (upper panel, left lane) or no receptor (293, lower panel, left lane) were used as controls for antibody specificity.

(b and c) Cells stably expressing FLAG-MOR, HA-D MOR, or both receptors were fed antibody to the extracellular epitope tag of the receptor and examined for receptor distribution following morphine treatment (5  $\mu$ M, 30 min). (b) MORs were distributed primarily on the cell surface in cells expressing only MOR, whereas D MORs were redistributed to endocytic vesicles. (c) In cells coexpressing both receptors, not only D MORs but also MORs were redistributed to endocytic vesicles, with a significant number of vesicles showing colocalization of both receptors (yellow in right panel).

and endocytosis, would have lower RAVE values than morphine.

These observations have led us to propose that drugs with high RAVE values, such as morphine, have an enhanced propensity to produce the adverse effects associated with prolonged drug exposure, precisely because

they signal through the receptor for aberrantly long periods of time. It follows that if prolonged signaling at MOR contributes to the development of tolerance and withdrawal, then molecular events that reduce this prolonged signaling, such as desensitization and endocytosis, would reduce the development of these side effects.



**Figure 2. D MOR Affects the Trafficking of MOR in Hippocampal Neurons**

Three-week-old hippocampal cultures were transfected with FLAG-MOR, HA-D MOR, or both receptors. Neurons were then examined for receptor distribution following antibody feeding and morphine treatment (5  $\mu$ M, 30 min). MORs in neurons expressing only this receptor were distributed primarily on the cell surface (upper left panel). D MORs were rapidly redistributed to endocytic vesicles upon morphine activation (upper right panel). In neurons that coexpressed MOR and D MOR, both receptors were redistributed to endocytic vesicles following activation by morphine (lower panels: left, MOR; right, D MOR).

Consistent with this hypothesis, we have recently demonstrated that enhancing morphine-induced endocytosis by receptor mutation reduces the development of tolerance and withdrawal in a cell culture model as assessed by superactivation of the cAMP pathway (Finn and Whistler, 2001). In addition, methadone, an agonist that promotes endocytosis of the wild-type MOR, produces less superactivation in our cell culture model, consistent with its reduced RAVE value. Receptor mutations that block methadone-induced endocytosis, thus increasing the RAVE value of methadone, exacerbate cellular withdrawal. Furthermore, previous studies in animal models have provided evidence that agonists that promote receptor endocytosis have reduced propensity for causing tolerance and dependence when they are administered at equi-analgesic doses (Duttaroy and Yoburn, 1995; Rezvani et al., 1983; Stevens and Yaksh, 1989).

Recently, several groups have reported dimerization of GPCRs (Angers et al., 2000; Lee et al., 2000) including opioid receptors (Jordan and Devi, 1999). In fact, heterodimerization of opioid receptors has been shown to alter opiate ligand properties (Jordan and Devi, 1999) and affect receptor trafficking (Jordan et al., 2001). Here we report that opioid receptors can oligomerize in our cell culture system and affect trafficking of the receptors. We have used this observation to begin to explore what role receptor oligomerization might play in ligand-mediated signaling from opioid receptors and whether receptor oligomerization can alter the RAVE value of particular receptor agonists. These studies have allowed us to address the RAVE hypothesis both in cell culture systems and in animal models of behavior, and have provided in vivo evidence that endocytosis of the MOR can reduce the development of tolerance to morphine in a rat model of opiate tolerance.

## Results

### The MOR Forms Oligomers with a D MOR Receptor

First, we examined whether the wild-type MOR could form heterodimers with a previously described mutant MOR, D MOR, that has altered trafficking properties (Finn and Whistler, 2001; Whistler et al., 1999). The D MOR receptor is a chimera in which the cytoplasmic tail of the MOR has been replaced by the corresponding residues of the delta opioid receptor. This confers upon this receptor a gain-of-function phenotype whereby morphine can promote receptor phosphorylation, arrestin recruitment, and endocytosis (Whistler et al., 1999). Human embryonic kidney (HEK) 293 cell lines stably transfected with both a FLAG-tagged MOR and an HA-tagged D MOR were generated. Cells were treated with morphine (MS) or etorphine (ET) or left untreated (NT), and the FLAG-tagged MORs were immunoprecipitated. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-FLAG antibodies as a control, or anti-HA antibodies to detect D MOR and hence dimers/oligomers. The D MOR receptor efficiently coimmunoprecipitated with the MOR (Figure 1a). Dimerization did not appear to be ligand-dependent (Figure 1a).

### Receptor Oligomerization Alters MOR Trafficking Properties

We next assessed whether the heterodimerization of MOR and D MOR could affect the trafficking of the receptors using immunocytochemical methods. The double stable cell lines above were fed antibodies to the N-terminal extracellular tags of the FLAG-MOR and HA-D MOR. Cells were treated with morphine and fixed. Cell lines expressing only one receptor, either FLAG-



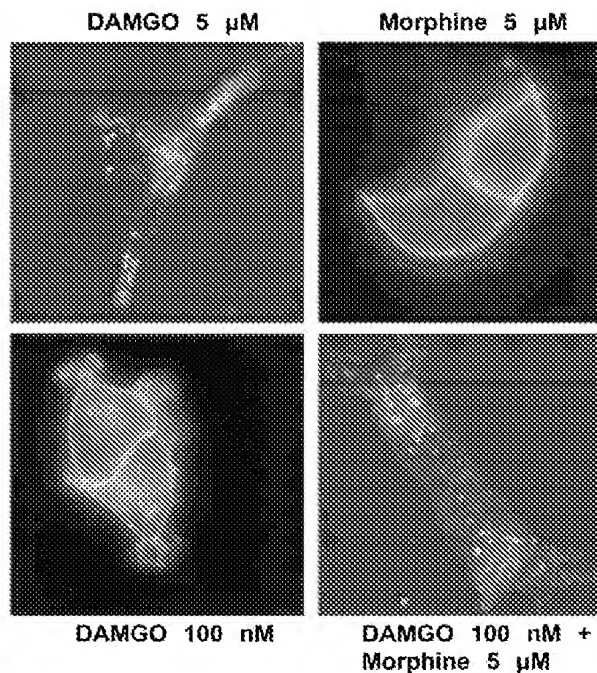
MOR or HA-D MOR, were treated analogously as controls. Cells were then permeabilized and stained with fluorescent conjugated secondary antibodies. In this way, only receptors that were initially on the cell surface were detected. As expected, cells expressing only MOR failed to show significant receptor endocytosis when treated with morphine, whereas the D MOR receptor efficiently internalized (Figure 1b). In contrast, the MOR in the cell line that coexpressed D MOR underwent significant endocytosis in the presence of morphine, with a substantial number of vesicles showing colocalization of both receptors (Figure 1c). We called this phenomenon "dragging" because it appeared that the D MORs could drag the MORs into the cell in response to morphine, presumably because these receptors were making heterodimers (Figure 1a).

#### The D MOR Affects MOR Trafficking in Cultured Neurons

To ensure that this phenomenon was not an artifact of the HEK 293 cell model, we examined whether dragging also occurred in cultured neurons. Hippocampal neuron cultures were prepared from rat and were allowed to mature for three weeks. Cultures were then transfected with FLAG-MOR alone, HA-D MOR alone, or both receptors. Cultures were fed anti-FLAG and/or anti-HA antibodies, then treated for 30 min with morphine. As previously reported (Whistler et al., 1999), neurons expressing MOR alone expressed receptor primarily on the plasma membrane following morphine treatment (Figure 2, upper left panel). In contrast, cells expressing D MOR alone showed efficient redistribution of receptors to endocytic vesicles following activation by morphine (Figure 2, upper right panel). Importantly, in neurons that expressed both receptors, both the D MOR and the wild-type MOR were redistributed to endocytic vesicles following activation by morphine (Figure 2, lower panels). These results demonstrate that the D MOR receptor can drag the MOR into neurons.

#### DAMGO Facilitates Morphine-Induced Endocytosis of MOR

We have also observed that wild-type MORs can homodimerize with one another just as they heterodimerize with D MOR (data not shown). Because of this observation, we designed an experiment to ask whether we could facilitate receptor dragging using the pharmacology of different MOR agonists. DAMGO, a hydrolysis-resistant derivative of enkephalin, promotes robust endocytosis of the MOR (Keith et al., 1996) and has an affinity for the MOR similar to that of morphine (Raynor et al., 1994). These observations allowed us to address whether a DAMGO-occupied MOR could drag a morphine-occupied MOR into the cell. HEK 293 cells expressing only wild-type MOR were treated with a saturating dose of DAMGO (5  $\mu$ M) or a saturating dose of morphine (5  $\mu$ M). As expected, cells treated with DAMGO showed robust endocytosis of receptor (Figure 3, upper left), while cells treated with morphine showed little endocytosis of receptor (Figure 3, upper right). When we treated the same cells with a nonsaturating dose of DAMGO (100 nM), there was significantly less

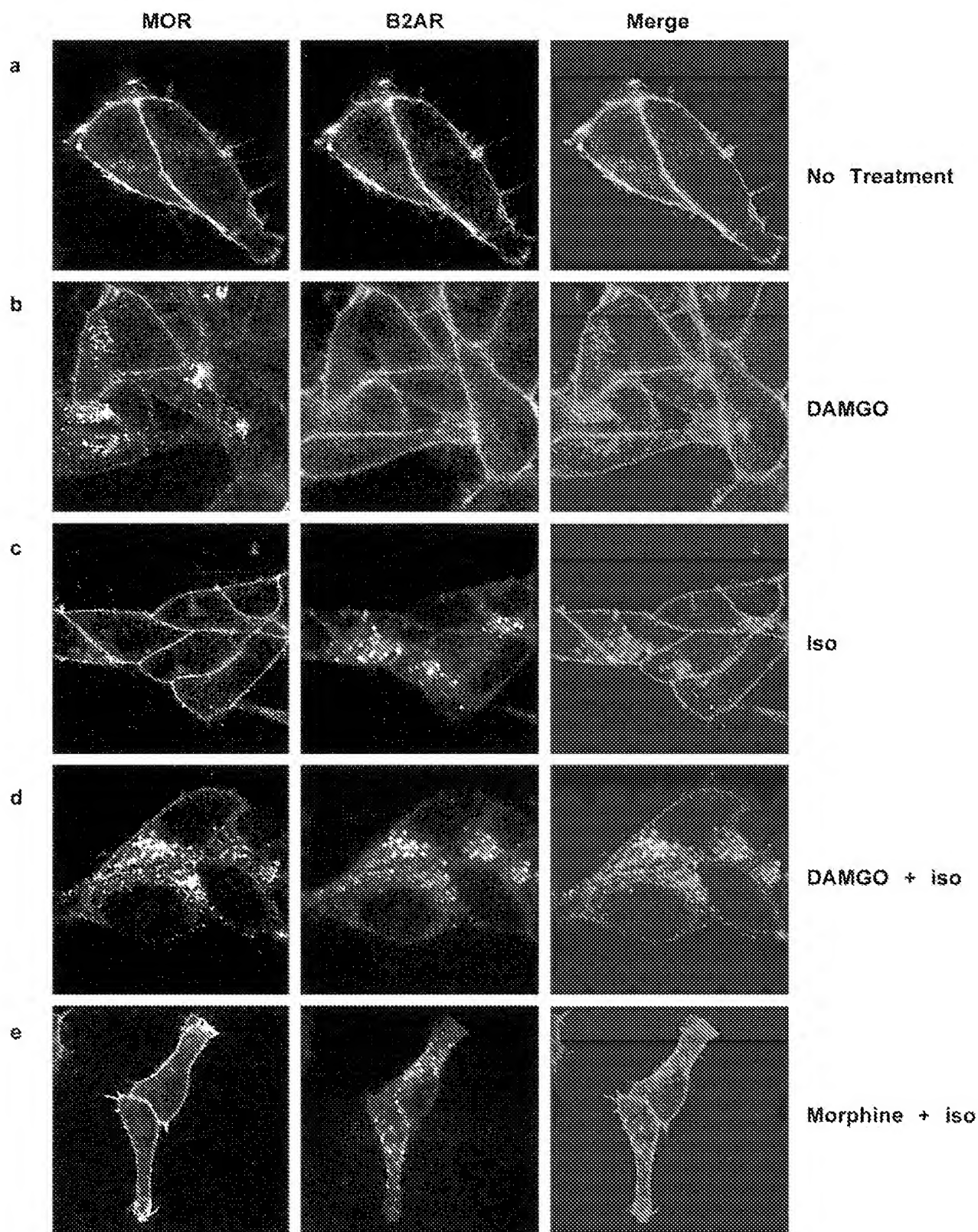


**Figure 3. DAMGO Facilitated Morphine-Induced MOR Endocytosis** HEK 293 cells expressing only wild-type MOR were analyzed for receptor distribution following treatment with various agonists. A saturating concentration of DAMGO (5  $\mu$ M, 30 min) promoted robust endocytosis of MOR (upper left panel), whereas morphine at the same dose had little effect on receptor distribution (upper right panel). A subsaturating dose of DAMGO (100 nM) caused reduced endocytosis compared to a saturating dose (lower left panel). However, this subsaturating dose of DAMGO (100 nM), when administered concurrently with a saturating dose of morphine (5  $\mu$ M), facilitated robust endocytosis of the MOR (lower right panel).

receptor endocytosis (Figure 3, lower left), presumably because of low receptor occupancy.

We next asked whether these few DAMGO-occupied receptors could drag morphine-occupied receptors into the cell. To accomplish this, we treated cells simultaneously with the nonsaturating dose of DAMGO (100 nM) and a saturating dose of morphine (5  $\mu$ M). Assuming all receptors are monomers, one would predict that the saturating dose of morphine would act as an antagonist for the submaximal endocytosis induced by the subsaturating dose of DAMGO. Remarkably, cells treated in this way showed robust receptor endocytosis (Figure 3, lower right). We attribute this phenomenon to the ability of a few DAMGO-activated receptors to drag several morphine activated receptors into the cell.

These results suggest that the receptors are making oligomers rather than simple dimers and that a single DAMGO-occupied receptor in an oligomeric complex with other morphine-occupied receptors is sufficient to recruit the endocytic machinery and facilitate oligomer internalization. Alternatively, it suggests that the few DAMGO-activated receptors in the cell are bringing a high local concentration of the endocytic machinery, in particular arrestin, to the morphine-activated receptors. We have demonstrated previously that overexpression of arrestin can facilitate morphine-induced endocytosis of wild-type MOR (Whistler and von Zastrow, 1998). To



**Figure 4.** The B2AR Did Not Drag Morphine-Activated MORs into Cells

Cells stably expressing FLAG-MOR and HA-B2AR were fed antibody to the extracellular epitope tags of the receptors and examined for receptor distribution following various agonist treatments (all 5  $\mu$ M, 30 min).

(a) MOR and B2AR were distributed primarily on the cell surface in the absence of agonist.

(b) DAMGO facilitated MOR endocytosis but not B2AR endocytosis.

(c) Isoproterenol facilitated B2AR endocytosis but not MOR endocytosis.

(d) In the presence of both agonists, both receptors were efficiently internalized and showed significant colocalization (yellow in merge panel).

(e) Isoproterenol-activated B2AR receptors failed to drag morphine-activated MORs into the cell.

differentiate between these possibilities, we examined whether an unrelated GPCR, the  $\beta$ -2 adrenergic receptor (B2AR), when activated, could facilitate the heterologous endocytosis of the morphine-activated MOR.

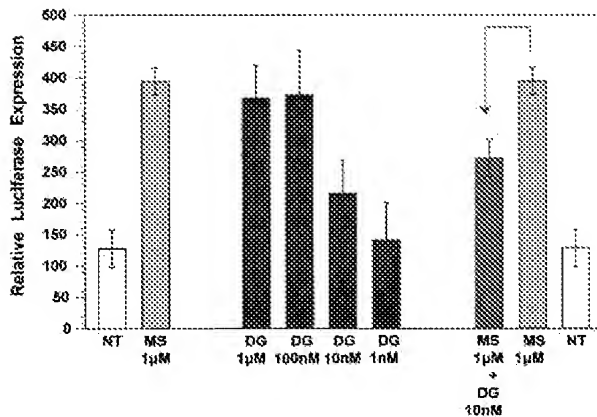
HEK 293 cells were generated that stably expressed both the FLAG-tagged MOR and an HA-tagged B2AR. Cells were incubated with antibodies to both epitope tags to label cell surface receptors, treated with various agonists or agonist combinations, then stained for both receptors. Both receptors were expressed primarily on the cell surface in the absence of agonist (Figure 4a). As expected, DAMGO promoted endocytosis of the MOR but not B2AR (Figure 4b), while the B2AR receptor agonist isoproterenol (iso) promoted B2AR receptor but not MOR endocytosis (Figure 4c). In the presence of both agonists, both receptors were efficiently internalized (Figure 4d). However, isoproterenol-activated B2ARs were not able to drag morphine-activated MORs into the cell (Figure 4e). These results suggest that heterologous activation of the B2AR receptor and its consequent membrane recruitment of arrestin (data not shown) is insufficient to promote the endocytosis of nearby MORs. Hence, it is likely that receptors must be in an oligomeric complex in order for dragging to be efficient.

The precise mechanism whereby a small number of DAMGO-occupied receptors facilitate endocytosis of the morphine-activated receptors remains an intriguing question, and one that we are actively pursuing. Nevertheless, knowing the mechanism by which this dragging is accomplished was not necessary for us to assess the functional consequences of "dragging by pharmacology."

#### DAMGO Reduces Morphine-Induced cAMP Superactivation

Chronic morphine treatment of animals, as well as cells in culture, produces a compensatory upregulation of the cAMP pathway (Sharma et al., 1975; Bonci and Williams, 1997; Avidor-Reiss et al., 1996), an effect that has been studied as a cellular hallmark of opiate withdrawal that we have also demonstrated contributes directly to a form of cellular tolerance (Finn and Whistler, 2001). Previously we have demonstrated that receptor endocytosis can reduce this compensatory upregulation (Finn and Whistler, 2001). Hence, we predicted that receptor dragging could reduce superactivation in our cell culture model. We assessed the functional consequences of dragging by pharmacology in a previously described cell line that expresses MOR and a CRE-luciferase reporter gene (Finn and Whistler, 2001). As expected, morphine induced substantial superactivation (Figure 5, gray bar). DAMGO also induced superactivation in this cell line in a dose-dependent manner (Figure 4, black bars), despite its ability to promote receptor endocytosis. We attribute this to DAMGO's enhanced potency (and hence greater numerator value in its RAVE) compared to that of morphine (Avidor-Reiss et al., 1996). Remarkably, a low dose of DAMGO (10 nM), which alone produced little superactivation, substantially reduced superactivation when it was administered simultaneously with a superactivation-inducing dose of morphine (1  $\mu$ M) (red bar).

Taken together, these data demonstrate that a second, endocytosis-promoting agonist can facilitate morphine-induced receptor endocytosis, consequently re-



**Figure 5. DAMGO Reduced Morphine-Induced cAMP Superactivation**  
Cells stably expressing MOR and a CRE-luciferase reporter gene were treated chronically (14 hr) with morphine, DAMGO, or both drugs, and superactivation of the cAMP pathway was assessed relative to untreated cells. Morphine (1  $\mu$ M) caused pronounced superactivation (gray bars) (Finn and Whistler, 2001). DAMGO also caused superactivation in a dose-dependent manner. A dose of DAMGO that caused little superactivation (10 nM) when administered concurrently with the superactivation-inducing dose of morphine (1  $\mu$ M) reduced the morphine-induced superactivation (red bar).  $P < 0.01$ , two-way ANOVA, Tukey's post test.

ducing the RAVE value of morphine and reducing the compensatory adaptive cellular changes that lead to upregulation of the cAMP pathway, at least in a cell culture model. Importantly, these observations allowed us to design experiments to examine the role of receptor endocytosis in the development of tolerance in an intact animal.

#### DAMGO Facilitates Morphine-Induced Endocytosis in Rat Spinal Cord Neurons

To begin these studies, we first assessed whether we could facilitate morphine-induced endocytosis of the MOR using a low dose of DAMGO *in vivo*. Rats were implanted with an intrathecal catheter through which either an acute injection of agonist could be given or chronic drug could be administered by an osmotic mini pump. We first examined the effects of a single acute injection of morphine and DAMGO on analgesia and endocytosis. Consistent with previous studies (Advokat, 1993; Malmberg and Yaksh, 1992; Trafton et al., 2000), an acute high dose of both DAMGO (0.3 nmoles) and morphine (30 nmoles) produced profound analgesia (Figure 6a). Following the behavioral testing, these animals were sacrificed and the distribution of MORs was examined using immunohistochemical staining. Neurons from the lamina II of the spinal cord dorsal horn were examined because they play an important role in pain transmission (Yaksh, 1999). MOR was detected in numerous endosomes throughout the cell body of the lamina II neurons of rats treated with 0.3 nmoles DAMGO (Figure 6b, upper left panel), indicative of pronounced receptor endocytosis. In contrast, the MORs in lamina II neurons of the rats treated with 30 nmoles of morphine, which showed equivalent analgesia in the behavioral assay (Figure 6a), were primarily on the cell membrane (Figure 6b, upper right panel).

We next assessed whether we could facilitate mor-

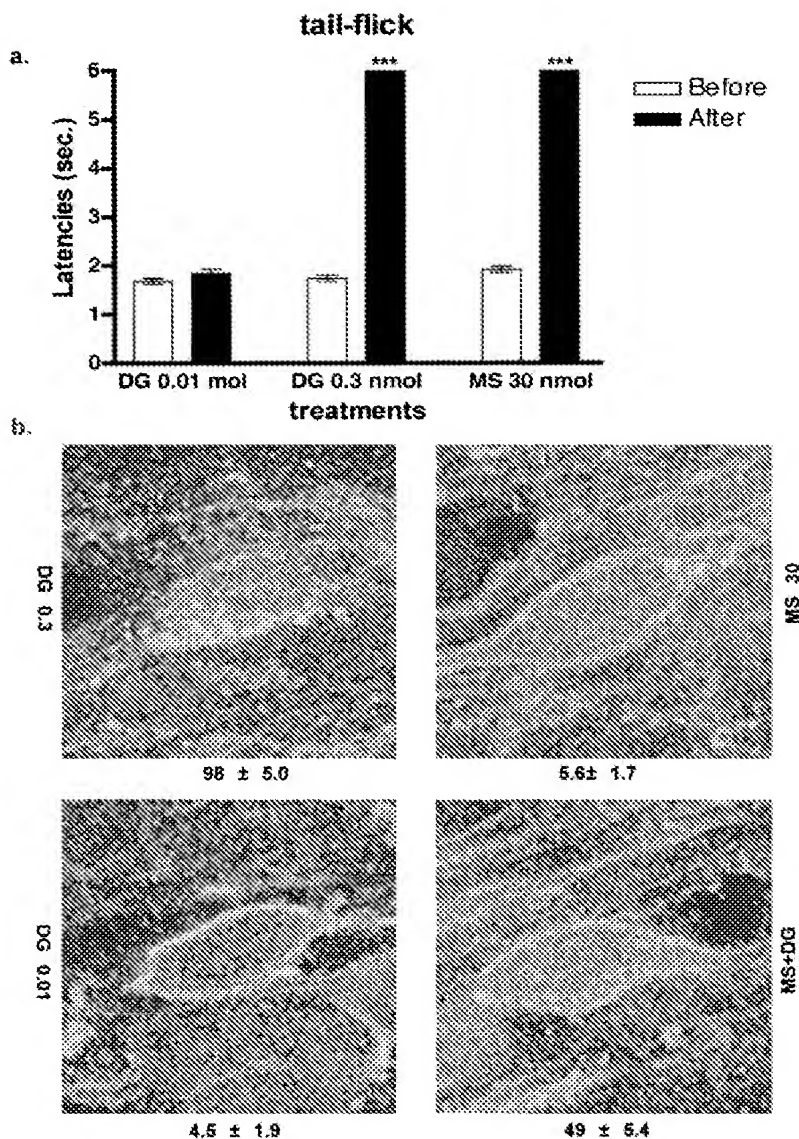


Figure 6. DAMGO Facilitated Morphine-Induced MOR Endocytosis in Rat Spinal Cord Rats (4–6 per group) were implanted with an IT catheter through which acute doses of drug were administered.

(a) Analgesia. Tail flick latency was tested before and 30 min after drug administration. 0.3 nmoles of DAMGO or 30 nmoles of morphine produced significant analgesia (\*\* $p < 0.001$ ), whereas 0.01 nmoles of DAMGO had no analgesic effect ( $p > 0.05$ ) as shown by Student's *t* test.

(b) Endocytosis. Immediately following the behavioral test, rats were perfused and MOR distribution was assessed by immunohistochemical staining of MORs in the lamina II neurons of the spinal cord proximal to the catheter. MORs were redistributed to endocytic compartments following treatment with 0.3 nmoles of DAMGO. Little endocytosis was observed following treatment with the equi-analgesic dose of morphine (30 nmoles), or with the subanalgesic dose of DAMGO (0.01 nmoles). However, MORs in rats treated simultaneously with 0.01 nmoles of DAMGO and 30 nmoles of morphine were redistributed to endocytic vesicles. Quantification of vesicles is listed below each image and was achieved by encoding the slides and having a second party count vesicles from a center section of a Z stack for at least 8 cells per condition from 2 rats per condition.

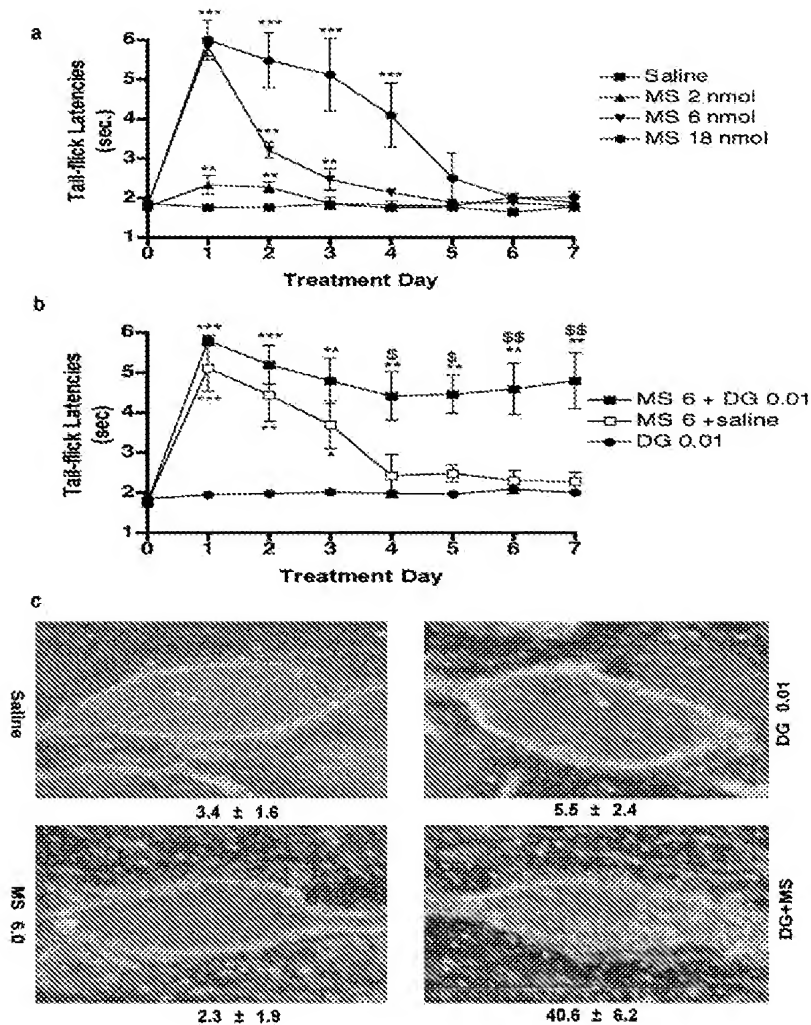
phine-induced endocytosis of MOR using DAMGO in vivo. A very low dose of DAMGO was chosen to avoid confusion due to DAMGO-induced endocytosis. Consistent with previous reports (Trafton et al., 2000), DAMGO, at a dose of 0.01 nmoles, produced neither significant antinociception in the tail-flick assay (Figure 6a) nor detectable MOR endocytosis in lamina II neurons (Figure 6b, lower left panel). However, this low dose of DAMGO, when administered concurrently with 30 nmoles of morphine, elicited a remarkable endocytosis in the spinal cord neurons (Figure 6b, lower right panel). These results clearly demonstrate that DAMGO and morphine differentially regulate MOR trafficking in the spinal cord and that DAMGO can facilitate morphine-induced endocytosis in vivo, thereby altering the RAVE value of morphine.

#### DAMGO Reduces Morphine Tolerance

Using these observations, we designed a set of experiments to examine whether alteration of the RAVE value of morphine by DAMGO-mediated dragging would affect the development of morphine tolerance. We first

examined the time course of intrathecal morphine tolerance. As above, rats were implanted with an intrathecal catheter through which chronic morphine at several doses was administered by mini pump. As shown in Figure 7a, three different doses of morphine produced a significant antinociceptive effect for the first few days. However, antinociception was gradually reduced during continuous exposure to morphine and was eventually lost completely over 7 days, indicating that the rats had developed tolerance to morphine. To examine whether the interaction between DAMGO and morphine, which stimulated endocytosis of MOR acutely (see Figure 6b), could reduce the development of tolerance to chronic morphine, we designed the following experiment. Rats were implanted with a Y-shaped intrathecal catheter. One arm of the Y was connected to a mini pump through which chronic morphine or saline was administered. Either saline or a subanalgesic, subendocytic dose of DAMGO (0.01 nmoles; see Figures 6a and 6b) was administered twice daily through the other arm of the catheter. Twice daily injection of 0.01 nmoles of DAMGO produced no analgesia in the rats receiving saline from





their mini pumps (Figure 7b, closed circles), consistent with the inability of this dose of DAMGO to produce antinociception acutely (Figure 6a). Rats receiving morphine chronically through their mini pumps and twice daily injections of saline through the catheter showed pronounced antinociception early in the experiment but developed tolerance to the effects of morphine within 4 days (Figure 7b, open squares). Rats receiving the same dose of morphine through their minipumps and also receiving twice daily injection of 0.01 nmoles of DAMGO through their catheters showed antinociception on day 1 comparable to that in the rats receiving saline injections. However, remarkably, these rats did not develop tolerance to morphine during the seven days of this experiment (Figure 7b, closed squares).

We hypothesized that the failure of the rats receiving both morphine and DAMGO to develop tolerance was a reflection of the ability of a low dose of DAMGO to alter the RAVE value of morphine by stimulating receptor endocytosis. To examine this possibility, we examined the distribution of the MORs in the spinal cord of the rats from the behavioral experiment. The distribution of MORs in spinal cord neurons of the rats receiving twice daily injection of 0.01 nmoles DAMGO and saline in the mini pump was indistinguishable from animals given only saline (Figure 7c, compare top two panels), con-

Figure 7. DAMGO Inhibited the Development of Morphine Tolerance

(a) Rats were implanted with an IT catheter and a time course of morphine tolerance development was assessed with daily tail flick latency testing before pump implantation (day 0) and for 7 consecutive days. Morphine was chronically infused at 2, 6, or 18 nmoles/hr. Morphine induced tolerance at all three doses. (b) Rats were implanted with a Y-shaped IT catheter. One arm of the catheter was attached to a mini pump that was prefilled with morphine and implanted subcutaneously. The other arm of the catheter was used for daily injection of DAMGO or saline. 0.01 nmoles of DAMGO/15  $\mu$ l or the same volume of saline were injected twice per day. Analgesia was assessed by tail flick latency test once per day 30 min following the second injection. The results were analyzed by two-way ANOVA followed by Bonferroni post-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus saline; \$  $p < 0.05$ , \$\$  $p < 0.01$  significantly different from MS 6 nmole plus saline group.  $N = 4-6$  per group, mean  $\pm$  SEM are shown). There was no significant difference between the MS 6 nmole plus DG 0.01 nmole group and the MS 6 nmole plus saline group for the first 3 days ( $p > 0.05$ ).

(c) Immunohistochemical staining of MORs in the lamina II neurons of the spinal cord from the rats in (b). MORs were primarily localized to the plasma membrane of neurons of rats treated with saline, morphine, or the low dose of DAMGO. However, pronounced MOR endocytosis was observed following coadministration of morphine with the low dose of DAMGO. Quantification was achieved by encoding the slides and having a second party count vesicles from a center section of a Z stack for at least 8 cells from 2 rats per condition.

tent with the results obtained with this dose of DAMGO acutely. The MORs in the spinal cord neurons of the rats given chronic morphine were also predominantly on the cell surface (Figure 7c, lower left panel), consistent with the results obtained with acute morphine in spinal cord neurons of this study (see Figure 6b, upper right panel) and other regions in previous studies (Keith et al., 1998; Stermini et al., 1996). In contrast, the MORs in the spinal cord neurons of the rats with a morphine mini pump who received twice daily injection of 0.01 nmoles DAMGO were distributed not only on the plasma membrane but also within intracellular compartments, suggesting that MORs in these rats were undergoing endocytosis in response to a low dose of DAMGO in combination with chronic morphine. Taken together, these results imply that a subanalgesic dose of a MOR agonist that promotes receptor endocytosis can facilitate the endocytosis of morphine-activated receptors in the cell, thereby decreasing the RAVE value of morphine and reducing the development of tolerance.

## Discussion

Here we show that a small, subanalgesic dose of DAMGO, an agonist that promotes endocytosis of the MOR, facilitates morphine-induced endocytosis *in trans*

and thereby lowers the RAVE value of morphine and reduces the development of tolerance. We propose that oligomerization of the MOR influences the endocytic properties of the receptor, and as a consequence of this altered endocytosis, the development of tolerance to morphine is reduced. Although we can not rule out the possibility that other mechanisms associated with the interaction of DAMGO and morphine could be affecting the development of tolerance to morphine, we favor the hypothesis that the rats treated with both drugs become less tolerant to the analgesic effects of morphine as a consequence of the decreased RAVE value of morphine. These results are consistent with our previous studies in cell culture that have demonstrated that increases in endocytosis reduce tolerance and withdrawal. However, these data provide *in vivo* evidence that suggests that alterations in the trafficking properties of the MOR in response to morphine can affect the development of tolerance in an animal model of behavior.

It is likely that tolerance to opiate drugs, as well as other compounds that target GPCRs, is mediated by a complex set of mechanisms. We have previously shown that tolerance to morphine in a cell culture model can occur by at least two distinct mechanisms depending on the endocytic and postendocytic properties of the receptor in response to morphine (Finn and Whistler, 2001). Furthermore, Yoburn and colleagues have demonstrated that opiate tolerance can occur by receptor density-dependent and -independent mechanisms depending on whether or not the agonist used promotes endocytosis (Stafford et al., 2001).

Tolerance to morphine can occur as a result of superactivation of the adenylyl cyclase signaling pathway (Sharma et al., 1975), which masks morphine's effect by altering the homeostatic baseline of the MOR expressing cells. Several groups have reported superactivation of the cAMP signaling pathway in response to chronic morphine treatment in brain regions implicated in addiction, including the locus coeruleus (Nestler, 1996), ventral tegmental area (Bonci and Williams, 1997), nucleus accumbens (Chieng and Williams, 1998; Terwilliger et al., 1991), amygdala (Terwilliger et al., 1991), and dorsal raphe (Jolas et al., 2000). Cellular changes occurring during cAMP superactivation include increased expression of certain adenylyl cyclases, PKA, and CREB (reviewed in Nestler, 2001; Williams et al., 2001). These adaptive cellular changes compensate for continued inhibition of adenylyl cyclase, and are functionally analogous since they serve to increase the amount of signaling through the cAMP pathway and thus subvert the effect of morphine. This cellular tolerance is clearly revealed upon removal of drug, whereby the superactivation manifests itself as withdrawal. Superactivation following drug withdrawal demonstrates that the MORs in these cells are still coupled to second messenger cascades when drug is present, and hence this form of tolerance would be receptor density-independent. This cellular tolerance is alleviated by receptor endocytosis (Finn and Whistler, 2001).

On the other hand, tolerance to morphine could also occur as a result of receptor desensitization or receptor downregulation. Tolerance mediated solely by receptor desensitization would lead to reduced receptor-mediated signaling without a loss of surface receptors. Several groups have reported reduced MOR-mediated signaling

in various brain regions following chronic morphine treatment, often without a concomitant loss in receptor number (Christie et al., 1987; Connor et al., 1999; Selley et al., 1997; Sim et al., 1996). Tolerance mediated by receptor downregulation would lead to reduced receptor-mediated signaling because of a loss of surface receptors. Several groups have reported that in some brain regions there is, in fact, a loss of receptors following prolonged morphine treatment (Abdelhamid and Takemori, 1991; Bernstein and Welch, 1998; Tao et al., 1998). However, in other regions, receptor number remains unchanged (De Vries et al., 1993; Simantov et al., 1984; Werling et al., 1989) or is even upregulated in tolerant animals (Brady et al., 1989; Gouarderes et al., 1990; Rothman et al., 1991; Tejwani et al., 1998). It is likely that all these mechanisms, and potentially others as well, contribute to opiate tolerance. Furthermore, although cellular mechanisms, including receptor number, desensitization, and homeostasis can contribute to tolerance, additional complex mechanisms involving alterations in neuronal circuitry are likely involved in the development of associative tolerance (Mitchell et al., 2000).

The observation that  $\beta$ -arrestin 2 knockout mice show reduced analgesic tolerance (Bohn et al., 2000) suggests that, in certain cell types, receptor desensitization may contribute directly to morphine tolerance, perhaps by serving as a first step toward receptor downregulation, although receptor number was not assessed in these animals. These data are consistent with the prevailing hypothesis that receptor desensitization contributes directly to tolerance. However, it is important to note that the endocytic trafficking of several classes of GPCR are likely also affected by the loss of  $\beta$ -arrestin in these animals, many of which may also be involved in pain transmission. Furthermore, the  $\beta$ -arrestin 2 knockout mice still demonstrate withdrawal from morphine, as assessed biochemically with cAMP superactivation. Hence, cellular tolerance is still occurring in these animals, even though behavioral tolerance is reduced. Clearly, the emerging picture of regional differences in the extent of chronic morphine-induced MOR desensitization (Sim et al., 1996; Sim-Selley et al., 2000), as well as regionally distributed splice variants differing in their cytoplasmic tails (Abbadie et al., 2000), promises to impart considerable complexity to the biochemical characterization of the processes of desensitization and superactivation in different brain regions.

Nevertheless, here we have demonstrated that increased MOR endocytosis in response to morphine can reduce the development of tolerance in an animal model. These results have important implications for the treatment of chronic pain. First, they suggest that agonists that promote endocytosis of the MOR might provide analgesics with reduced liability for tolerance. This is in contrast to the prevailing hypothesis that desensitization and endocytosis of the MOR contributes directly to tolerance by decreasing the number of functional receptors. It is important to note that agonists that promote desensitization of receptors are routinely discarded in drug discovery programs precisely because of this prevailing view. However, even without the development of new opiate analgesics, the results here suggest that the development of tolerance to morphine can be delayed by the coadministration of drugs that promote

endocytosis. In short, our results suggest that two drugs actually produce less tolerance than morphine alone.

## Experimental Procedures

### Cell Culture and Immunocytochemistry

Human Embryonic Kidney (HEK) 293 cells (American Type Culture Collection) were grown in DMEM (Gibco BRL) supplemented with 10% Fetal Bovine Serum (Hyclone). Mu opioid receptor and CRE-Luciferase (Promega) constructs were stably transfected using calcium phosphate coprecipitation, with single colonies chosen and propagated in the presence of selection-containing media. For immunocytochemistry, cells were grown on poly-lysine coated coverslips and incubated with 3.5  $\mu$ g/ml M1 anti-FLAG and/or 3.5  $\mu$ g/ml HA-11 antibody (Covance) for 30 min. Cells were then treated with agonist as specified, fixed in 4% formaldehyde in PBS, permeabilized in 0.1% Triton X-100 in Blotto, and stained. Cells stained for only one receptor type were stained with Texas red-conjugated Donkey anti-Mouse antibody (Jackson ImmunoResearch). Cells that were stained for both FLAG and HA tagged receptors simultaneously were first incubated with rabbit anti-IgG<sub>2b</sub> antibodies (Zymed) followed by staining with Texas red Donkey anti Rabbit antibody (Jackson ImmunoResearch) and FITC-conjugated Goat anti Mouse IgG<sub>1</sub> antibody (Boehringer). Images were acquired using a custom-configured inverted microscope (Prairie Systems, Madison, WI) with a Zeiss 63 $\times$  oil objective, or a Zeiss confocal with a 60 $\times$  oil objective.

### Immunoprecipitation

Cells were grown to 80% confluency in 10 cm dishes and treated with 5  $\mu$ M agonist for 30 min or left untreated. Cells were washed 2 $\times$  in PBS and lysed in NDM lysis buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5% n-dodecyl- $\beta$ -D-maltoside). Lysate was cleared by centrifugation at 10,000 rpm for 10 min at 4°C, and cleared lysate was immunoprecipitated with 40  $\mu$ l M2-conjugated sepharose (Covance) overnight at 4°C. Immunoprecipitates were extensively washed with NDM buffer followed by 2 washes with 10 mM Tris (pH 7.5). Receptors were deglycosylated with PNGase (NEB) in 10 mM Tris (pH 7.5) for 2 hr at 37°C, denatured with SDS sample buffer, and resolved by SDS-PAGE. Blots were blocked in Blotto, incubated with a biotinylated M2 anti FLAG antibody (1:250, Covance) for 2 hr, and developed with streptavidin overlay using ABC reagents (Vector laboratories) and ECL reagents (Amersham) as a control, or incubated with HA-11 antibody (1:1000 Covance) for 2 hr and HRP-conjugated Goat anti mouse (1:3000, Jackson ImmunoResearch) for 1 hr and developed with ECL reagents to detect oligomers.

### CRE-Luciferase Reporter Expression Assays

Cells were grown to confluency in 24 well plates. For acute experiments, cells were given drug for 4 hr and the fold inhibition of forskolin-stimulated luciferase activity measured. For chronic treatment experiments, cells were given drug for 14 hr, rinsed 3 times in drug-free media to initiate a withdrawal phase, then given 2  $\mu$ M forskolin for the 4 hr withdrawal phase, and luciferase activity measured. 14 hr was chosen after an initial time course of morphine-induced superactivation in MOR-expressing HEK293 cells demonstrated that superactivation at this time point was highly reproducible. For all treatment conditions, cells were rinsed once in PBS immediately prior to luciferase measurement. 100  $\mu$ l Cell Culture Lysis Reagent (Promega) was added to each well, a 20  $\mu$ l cell lysate aliquot was transferred to an opaque 96 well plate, 100  $\mu$ l substrate added per well using a Lucy 2 luminometer (Anthos), and light measurements collected. Data were exported to Microsoft Excel for compilation, and Graph-Pad Prism 3.0 for graphical display, nonlinear regression curve fitting, and subsequent statistical analyses.

### Animals

Male Sprague-Dawley rats (250–300 g, Simonsen Laboratories, Inc., Gilroy, CA) were housed individually in temperature-controlled rooms with a 12 hr light/dark cycle. Food and water were available ad libitum. All procedures used in this study were in agreement with the NIH Guide for the Care and Use of Laboratory Animals and were

approved by the Animal Care and Use Committee at Gallo Center of the University of California, San Francisco.

### Preparation and Implantation of Intrathecal (IT) Catheters

Catheter implantation was performed according to methods of Yaksh with minor modifications (Yaksh and Rudy, 1976; Yaksh and Stevens, 1986). Two types of catheters were prepared depending on the regimen for test drug delivery. For the morphine alone groups (and the saline only controls), a 3 cm length of polyethylene tubing, PE-60, was connected to an 8 cm length of PE-10 tubing by heating. For the chronic morphine plus DAMGO/saline groups, a Y-shape catheter was prepared. For catheter implantation, rats were anesthetized with isoflurane and placed on a stereotaxic device with the head flexed forward. The PE-10 catheter was inserted into the spinal subarachnoid space through an incision in the atlanto-occipital membrane and advanced caudally extending to the lumbar enlargement of the spinal cord. After implantation of IT catheters, rats were returned to their home cages and allowed 7 days to recover from surgery. Those rats with normal motor function were implanted with a subcutaneous mini-osmotic pump (Model 2001, DURECT Corp., Cupertino, CA) that had been prefilled with morphine or saline on the dorsal part of neck under light isoflurane anesthesia.

### Drug Treatments

DAMGO and morphine sulfate were purchased from Sigma (St. Louis, MO) and dissolved in 0.9% physiological saline. The test drug was delivered via either single injection or chronic infusion. Morphine or saline was infused via mini-osmotic pump at a constant rate of 1  $\mu$ l/hr. DAMGO or saline was injected through one arm of the Y-shape catheter at 15  $\mu$ l volume.

### Antinociception Test

Rats were tested for antinociception using the radiant heat tail-flick procedure. The light intensity was adjusted to achieve base-line latencies of 1.5 to 2 s; a maximum latency of 6 s was set as the cutoff time to minimize damage to the tail. For the morphine alone group and the saline controls, the animals were tested by tail-flick once a day for 7 days following implantation of the mini-osmotic pump. For the morphine plus DAMGO and morphine plus saline groups, following mini pump implantation, rats were administered DAMGO or saline via the other arm of the Y-shape catheter twice a day for 7 days at 9:00 AM and 4:30 PM. Antinociception was tested by tail-flick 30 min after the afternoon administration. The behavioral data of antinociception were compared and statistically analyzed by two-way analysis of variance followed by Bonferroni post-test, where  $P < 0.05$  was considered significant.

### Immunohistochemistry

The rats were anesthetized with intramuscular ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (12 mg/kg) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer immediately following the tail-flick test at day 7 following pump implantation. The segment of spinal cord proximal to the tip of the catheter was dissected out, post-fixed overnight in the same fixative, and then transferred to a 30% sucrose buffer solution. Sagittal sections (30  $\mu$ m) were cut on a freezing microtome, preincubated in PBT solution (0.1 M phosphate buffer + 0.2% BSA and 0.2% Triton X-100) for 30 min, blocked in 5% normal goat serum in PBT solution for another 30 min, and then incubated in a rabbit anti-MOR antibody (DiaSorin, Stillwater, MN) at a 1:5000 dilution and mouse anti-NeuN antibody to identify the neurons in the section (Mullen et al., 1992) (Chemicon International, Temecula, CA) at 1:300 overnight at 4°C. Sections were extensively washed with PBT and incubated in Cy-3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) both at a 1:600 dilution for 2 hr at room temperature. The sections were then washed and mounted on slides. MOR distribution was examined with a Zeiss confocal microscope using a 60 $\times$  oil immersion objective. For quantification, slides from at least two different rats for each condition were stained by one researcher and encoded, and vesicles were counted blind by a second individual from the middle section of at least 8 cells per condition. Following compilation of vesicle counts, the code was broken.

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